THE FREE AMINO ACID CONTENT OF CERUMEN*

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In the course of a study of diseases involving the external auditory canal, (1, 2) an investigation of the distribution of free amino acids in defatted cerumen was begun to determine possible qualitative differences in the distribution of free amino acids in normal cerumen and secretion obtained from diseased external auditory canals.

Differences in amino acid composition between normal and other pathological states have been found. Roberts and Frankel (3) using the method of paper partition chromatography have demonstrated differences in the amino acid composition of neoplastic mouse tissue compared to normal mouse tissue. In a similar manner Roberts and Tishkoff (4) demonstrated that the amino acid composition of mouse epidermis varied in different phases of growth. Dent (5) extensively studied the amino acid composition of urine in the Fanconi syndrome.

Previous investigations of cerumen have been few and concern themselves almost entirely with the lipid fractions (6, 7). Nakashima (8), in publishing the most complete examination of cerumen to date, found the following constituents: cerotic acid, cholesterol, hexone bases, neurostearic acid, "a bitter substance," an acid $C_{17}H_{34}O_2$, a substance $C_8H_{14}NO_2$, and the amino acids, arginine, cystine, histidine, lysine, proline, and tyrosine.

METHODS AND MATERIALS

Pooled samples of normal cerumen obtained from healthy individuals were stored in a deep freeze refrigerator maintained below −15°C. Random samples weighing from 1 to 4 grams were prepared for study by refluxing the crude cerumen with 20 cc. of a 95% alcohol-ether mixture (3:1) for thirty minutes on a steam bath. Peroxide-free ether and redistilled solvents were used throughout. Re-extraction in the same manner with two additional portions of alcohol-ether essentially removed all of the extractable lipids. The defatted residue was then dried for ten minutes in a vacuum oven at 60°C. In various determinations the fat free residue represented 15–30% of the weight of crude cerumen.

Determinations were made by the two-dimensional paper chromatographic method (9, 10). Samples were prepared by extracting the defatted residue (40–60 mgm. samples) with 3 cc. of aqueous alcohol in a small glass homogenizer. Alcohol concentrations of 75, 80, 85, 90 and 95 per cent were used in various experiments. The homogenate was centrifuged and the residue was twice washed and centrifuged with 1.5 cc. portions of aqueous alcohol. The combined extract and washings were evaporated to dryness; the residue was taken up in three drops of distilled water and an aliquot of extract equivalent to 10–20 mgm. of defatted cerumen was spotted on large sheets of Whatman's No. 1 filter paper. Samples were prepared with and without H_2O_2 and ammonium molybdate treatment in order to be

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able to demonstrate the presence of cysteine as cysteic acid and methionine as methionine sulfone.

In several experiments the evaporated aqueous alcoholic extract was taken up in 3 drops saturated picric acid solution, centrifuged in small capillary tubes and the supernate spotted on filter paper sheets. This was done in an attempt to remove interfering substances and was found to be unsuccessful. Aliquots equivalent to 5–10 mgm. of defatted cerumen were hydrolyzed with 3 cc. of 6 *N* HCl in a sealed tube at 110°C. for 18 hours. The hydrolysate was evaporated to dryness, stored over NaOH pellets overnight, and then spotted on paper as described above.

The chromatograms were developed using water-saturated phenol and lutidine. Ten drops of concentrated NH_4OH were placed on the floor before closing the phenol chromatography box and ten drops of diethylamine were likewise added in the lutidine run. Water in a shallow dish was placed in each box to insure a H_2O saturated atmosphere. The phenol papers were dried overnight at room temperature in front of a fan. The chromatograms were developed in a constant temperature room maintained at 25 ± 1°C. The position of the spots was made visible by spraying the sheets with a 0.1% solution of ninhydrin in n-butanol. Duplicate, and in some cases triplicate, chromatograms were made. Aliquots of the same sample invariably demonstrated the same pattern of amino acids.

The one dimensional chromatographic technic described by Dent (5) and used by Tishkoff (11) for the fractionation of liver extract was used for the isolation of individual amino acids. The alcoholic extract from 300 mgm. of defatted cerumen was applied to the paper square in a row of spots along one edge; each spot containing an amount equivalent to 21 mgm. of fat free cerumen. After development in phenol, the two end strips were cut out and sprayed with ninhydrin. Locating the region of the desired amino acid on the two end strips enabled one to cut out and elute with distilled water the zone of paper containing the desired amino acid plus some adjacent contaminants.

Final separation was achieved by spotting the evaporated eluate as before and developing the chromatograms with lutidine. Treating the end strips with ninhydrin enabled one to locate a zone containing the desired amino acid free from the adjacent contaminants. The amino acid is eluted from this zone and its identity was established by further color and chromatographic tests.

The amino acids were identified by their relative positions on the filter paper and by comparison with a reference chromatogram prepared by Dent. In certain instances, known amino acids were added to the extract to check the position of a particular amino acid. Portions of the chromatogram could be reproduced with mixtures of known amino acids. Failure to detect a particular amino acid is taken to mean that the quantity of that amino acid was below the limit of sensitivity of the chromatographic method and did not necessarily indicate a complete absence of the amino acid.

RESULTS

When 75% alcohol was used for the extraction, considerable difficulty was encountered in the initial experiments in obtaining a good chromatogram with well separated and well defined spots. Chromatograms of several different samples of defatted cerumen showed spots bunched near the origin having not moved their normal distances in either the phenol or the lutidine direction. Although an aliquot of 20 mgm. produced spots of the optimum intensity, varying the aliquot from 5 to 45 mgm. had no effect on the general configuration of spots. Non-specific ninhydrin positive material of considerable intensity appeared as streaks in the phenol direction close to the origin. From the appearance of these streaks and from their influence on the chromatogram it appeared likely that they were of the nature of conjugate carbohydrates and polypeptides.

Hydrolysis of the 75% alcohol extract (10 mgm. aliquot) with 6 *N* HCl resulted in a well defined chromatogram with the disappearance of the ninhydrin-positive streaks. In addition, many amino acid spots appeared; among them were aspartic acid, glutamic acid, cysteic acid, glycine, serine, alanine, methionine sulfone, taurine, β-alanine, valine, phenylalanine, the leucines, arginine, lysine, proline, tyrosine, threonine, and glucosamine. Two spots of very weak intensity were unidentified and one other might have been histidine or γ-aminobutyric acid. In contrast the unhydrolyzed extract had many fewer spots; among them glutamic acid, aspartic acid, glycine, serine, alanine, threonine, valine, the leucines, and tyrosine could be identified with some degree of certainty.

Satisfactory chromatograms free from interfering substances were obtained as a result of the following series of experiments. Separate samples of defatted cerumen were extracted with 75%, 80%, 85%, 90%, and 95% aqueous alcohol

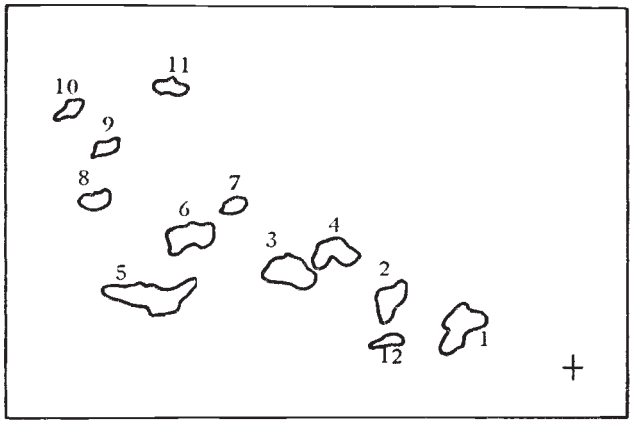

FIG. 1. Two dimensional paper chromatogram of 95% alcohol extract of defatted cerumen in phenol and lutidine.

solutions and the extracts chromatographed. The results showed that increasing the alcohol concentration decreased the intensity of ninhydrin-positive interfering substances. These substances were entirely absent from the chromatograph using 95% alcohol (Figs. 1 and 2). The definition and separation of the spots increased with increasing alcohol concentration and the R_f values increased in both the phenol and lutidine directions. All of the spots appearing on chromatograms of 75–80% alcohol extacts were also observed on chromatograms of 95% alcohol extracts although of less intensity. Glutamic acid appeared to be an exception to this in that the glutamic acid spot decreased in intensity with a decrease in alcohol concentration.

In the 95% alcoholic extracts the following amino acids were identified: aspartic acid, glutamic acid, glycine, serine, alanine, threonine, valine, the leucines, and tyrosine. The chromatogram of the above amino acids could be reproduced by a known mixture of these same amino acids. Two other spots appeared regularly on the chromatograms. One spot is chromatographically

identical with β-alanine. The addition of β-alanine to the extract increased the intensity of this spot. Isolation of this amino acid free from alanine, the nearest amino acid contaminant, was accomplished by the one-dimensional technique. Mixtures with known β-alanine behaved chromatographically as one substance. The differential color test with p-dimethylamino benzaldehyde was negative for citrulline. Alanine was isolated with one dimensional technique and proved to be chromatographically identical with the known amino acid.

The other spot appearing regularly had the position occupied by histidine on Dent's reference chromatogram. Attempts to characterize this substance were unsuccessful. The Pauly test (12, 13) for histidine was inconclusive due to interference of lutidine with the test.

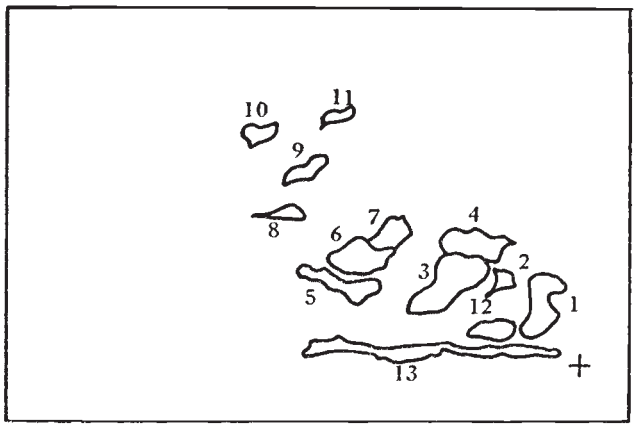

FIG. 2. Two dimensional paper chromatogram of 80% alcohol extract of defatted cerumen in phenol and lutidine.

1. Aspartic acid
2. Glutamic acid
3. Glycine
4. Serine
5. β-Alanine
6. Alanine
7. Threonine
8. Unidentified
9. Valine
10. Leucines
11. Tyrosine
12. Unidentified
13. Ninhydrin positive-interfering material

One other ninhydrin positive spot occasionally observed had the position of "underglutamic acid" reported by Roberts and Frankel (3). This material has been detected in a wide variety of tissues including the transplantable squamous cell carcinoma of mice and would appear to be a polypeptide containing at least 9 or 10 different amino acids. Attempts to isolate this material and to identify the constituent amino acids by acid hydrolysis from one batch of defatted cerumen were unsuccessful in that glutamic acid alone was obtained.

DISCUSSION

Roberts and Frankel (3) have investigated the free amino acid distribution in various normal and cancerous mouse tissues and Roberts and Tishkoff (4)

have studied the changes in free amino acid distribution during various stages of development of mouse epidermis. In light of the fact that some of the non-lipid residue of normal cerumen consists mainly of hair and epidermal debris, it is not surprising to find that the distribution of free amino acids found in this material most closely resembles that found in epidermal tissues. Whether these free amino acids are part of the cerumenous secretions *per se* or whether they come from the cellular debris cannot, of course, be determined here. However, several free amino acids usually observed in epidermis were not found in detectable amounts in the cerumen samples tested. Proline, hydroxyproline, and particularly taurine and the basic amino acids, arginine and lysine, were not found in this investigation. The significance of these observations is not as yet known.

The presence of β-alanine is worthy of note in that this amino acid is presumed to arise from the decarboxylation of glutamic acid and implies the presence of a glutamic decarboxylase at some point in the formation of crude cerumen.

Also of interest is the relatively large amount of interfering ninhydrin-positive substances that can be extracted with 75–80% alcohol solutions and which disappear on hydrolysis. Although increased numbers of amino acids are observed on acid hydrolysis of this material, the fact that a relatively large aliquot (10 mgm.) is necessary to produce a chromatogram of suitable intensity strengthens the possibility that considerable non-amino acid material is present, the most likely being carbohydrate in nature. Further study is needed to characterize the exact nature of this material as well as the two ninhydrin-positive spots occupying the positions of histidine and "underglutamic acid."

SUMMARY

The distribution of free amino acids in "normal" fat free cerumen was investigated by the method of paper partition chromatography. Extraction with 95% alcohol produced satisfactory chromatograms free from interfering substances. The following amino acids were identified: glutamic acid, aspartic acid, glycine, serine, alanine, threonine, valine, the leucines, β-alanine, and tyrosine. Two other ninhydrin-positive substances could not be identified with certainty.

ADDENDUM

Untreated cerumen extracted according to the procedure of Awapara (Arch. Biochem. **19**: 172, 1948) contained arginine, amino butyric acid and lysine besides the other amino acids present in the unhydrolyzed fat-free residue of ear wax. None of the fractions of this material examined showed any ninhydrin reactive substance characteristic of human cerumen.

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